METHOD AND COMPOSITION FOR AFFECTING REPRODUCTIVE SYSTEMS

This application claims the benefit of U.S. Provisional Applications Serial No. 60/142,308, filed July 1, 1999, and Serial No. 60/177,903, filed January 25, 2000.

Background of the Invention

Reproductive disorders in birds, rabbits, fish, reptiles and amphibians are common and can be difficult to treat. Some reproductive disorders, such as egg binding, are characterized by acute onset and cause a rapid deterioration in the health and physiological stability of the animal. Moreover, surgical and pharmacological interventions are often accompanied by high morbidity and mortality.

In birds, two of the most common clinically recognized reproductive disorders are egg binding and dystocia. Egg binding is the failure of an egg to pass through the oviduct at a normal rate. Dystocia (egg retention) defines a condition in which the developing egg is in the caudal oviduct and is either obstructing the cloaca or has caused oviduct tissue to prolapse through the oviduct-cloacal opening. Egg movement through the oviduct can stop at various locations. The most common anatomic areas for problems to occur are the caudal uterus, vagina and vaginal-cloacal junction. Budgerigars, canaries, finches, cockatiels and lovebirds most frequently have problems with dystocia. This is probably because the presentation of a palpable egg for more than a few hours in small birds is generally more serious than it is in larger birds. Dystocias are most critical in passerines and other small birds, many of which can survive only a few hours without aggressive therapy. Many hens with dystocia will attempt to lay another egg. Administration of medroxyprogesterone will temporarily stop ovulation, but there are side effects

and its use is controversial. Surgical correction, another alternative, is costly and dangerous.

Abnormally prolonged presence of an egg in the oviduct causes a multitude of complications in the hen. The severity of these complications depends on the species, the bird's previous health, the cause of the egg binding, the egg's location in the oviduct, and the time elapsed since egg development began. An egg lodged in the pelvic canal may compress the pelvic vessels and the kidneys, causing circulatory disorders and shock. An impacted egg may cause metabolic disturbances by interfering with normal defecation and mictruition, inducing ileus and renal dysfunction. Pressure necrosis may occur to all three layers of the oviduct wall and lead to rupture. Prolapse of the oviduct, impaction of the oviduct, and bacterial metritis may occur as sequela to dystocia or egg binding.

Another common reproductive problem in birds is chronic egg laying, which occurs when a hen lays eggs beyond the normal clutch size or produces repeated clutches regardless of the existence of a suitable mate or breeding season. This problem is particularly common in hand-raised hens that are imprinted on humans, and many highly domesticated psittacine birds like cockatiels, lovebirds and budgerigars are notorious chronic egg layers. Malnutrition and the progressive stress and physiologic demands of egg laying ultimately compromise the hen. Egg binding is common in hens that chronically lay eggs. Removing eggs from the hen effectively induces a form of double clutching and can exacerbate the problem. Medical therapy is directed to correcting any nutritional imbalances or reproductive tract abnormalities, and medroxyprogestrone injections can be used to temporarily interrupt the ovulatory cycle. However, depression, polyuria, weight gain, liver damage, immunosuppression and occasionally diabetes mellitus (especially in cockatiels) can occur with use of medroxyprogesterone. Egg laying may be stopped from two weeks to several months following therapy and repeat injections are often necessary, and the long term solution in these cases is a salpingohysterectomy, which is costly and dangerous.

Dystocia is also a common medical problem in reptiles, including snakes, turtles and lizards. Treatment options include physical manipulation, hormonal stimulation, percutaneous ovocentesis, and surgery. Common complications associated with manual palpation include oviductal rupture, oviductal prolapse, egg rupture, and even death. Furthermore, use of hormones that cause oviductal contraction on animals that have obstructive dystocia (as opposed to nonobstructive dystocia) can have detrimental consequences including egg fracture, oviduct rupture, hemorrhage and death. In lizards, spaying is often recommended to stop them from ovulating, thus preventing recurrent dystocias.

Dystocia is also a life-threatening disease in fish. In most cases, affected fish are either euthanized or the impacted egg mass must be surgically removed.

Most rabbit does that are allowed to undergo unrestricted estrus develop neoplasia of the reproductive tract or mammary tissues that is usually fatal. The only currently available technique to prevent these fatal neoplasias is ovariohysterectomy, preferably performed prior to a doe's first estrus.

The reproductive processes of other egg-producing organisms, such as amphibians, insects, arachnids, and plant and animal parasites, such as nematodes, while not typically targets for therapeutic intervention, offer opportunities for pest management via prevention of reproduction.

There is, therefore, a need for a safe and effective method for controlling the reproductive processes in birds, reptiles, fish, rabbits, amphibians, insects, arachnids, and plant and animal parasites and other egg-producing organisms. Such a method would be useful not only for population control, but to treat or prevent the onset of various disorders of the reproductive systems in these organisms.

Summary of the Invention

The present invention provides an immunogenic composition and a method for affecting the reproductive system of an oocyte-producing organism, preferably a bird, rabbit, fish, reptile, amphibian, insect, arachnid or an oocyte-producing parasite. One embodiment of the immunogenic composition, referred to herein as a protein vaccine, comprises a polypeptide comprising a zona pellucida protein or an immunogenic fragment thereof. Another embodiment of the immunogenic composition, referred to herein as a polynucleotide vaccine, comprises a polynucleotide comprising a nucleic acid sequence that encodes a polypeptide comprising a zona pellucida protein or an immunogenic fragment thereof.

The method for affecting the reproductive system of an organism comprises administering to the organism an immunogenic composition of the invention. The immunogenic composition, whether in the form of a protein vaccine or a polynucleotide vaccine, can be administered either prophylactically to prevent the occurrence, recurrence or onset of a reproductive system disease, disorder or condition, or therapeutically to treat a reproductive system disease, disorder or condition. When administered as an immunocontraceptive, the immunogenic composition causes temporary, reversible infertility in the organism. When administered as an immunosterilant, the immunogenic composition causes permanent, irreversible infertility in the organism. An immunogenic composition administered to control reproduction can optionally further function to treat or prevent one or more other reproductive disorders, diseases or conditions.

The immunogenic composition of the invention preferably comprises at least one of a mammalian zona pellucida protein or avian zona pellucida protein, but alternatively or in addition it can include a zona pellucida protein from other animals. A porcine zona pellucida (pZP) protein is a preferred mammalian zona pellucida protein, and a chicken zona pellucida protein is a preferred avian zona pellucida protein (aZP). Optionally, the immunogenic composition of the invention contains an immunological adjuvant.

The immunogenic composition is administered in a manner and an amount effective to affect one or more reproductive processes in the organism.

The invention further provides a zona pellucida protein, or a fragment thereof, conjugated to an immunogenic carrier protein, which can be used in an immunogenic composition to affect the reproductive system of an oocyte-producing organism according to the invention. Also provided is a dually or multiply functional immunogenic composition comprising a zona pellucida protein, or a fragment thereof, conjugated to at least one immunogenic carrier protein, wherein the immunogenic carrier protein is capable of generating an independently protective immune response.

Brief Description of the Drawings

Figure 1 shows one version of an oocyte purification apparatus.

Figure 2 shows chicken anti-pZP IgY titers over time for four pZP-vaccinated chickens and two control chickens.

Figure 3 shows egg production by week after pZP vaccination of chickens.

Detailed Description

The immunogenic composition of the invention comprises at least one zona pellucida protein or immunogenic fragment thereof, or at least one polynucleotide encoding a polypeptide comprising a zona pellucida protein or an immunogenic fragment thereof. The zona pellucida protein is preferably, but need not be, substantially pure. The zona pellucida protein or immunogenic fragment thereof can be a naturally occurring protein, a chemically or enzymatically synthesized protein, or a recombinant protein. The zona pellucida protein or immunogenic fragment thereof is preferably, but need not be, glycosylated. In a glycosylated zona pellucida protein (i.e., a zona pellucida glycoprotein), the glycosylation pattern is preferably equivalent to the glycosylation pattern found on a native (i.e., naturally occurring) glycoprotein. Naturally occurring zona pellucida protein used in this embodiment of the

immunogenic composition of the invention is not limited by the source of the protein, but is preferably isolated from pigs, birds, or fish. Zona pellucida proteins have been characterized in many different vertebrates, including, for example, pigs (e.g., E. Yurewicz et al., Biochim. Biophys. Acta, 1174, 211-214 (1993)), birds (e.g., Y. Takeuchi et al., Eur. J. Biochem., 260, 736-742 (1999); M. Waclawek et al., Biol. Reprod., 59, 1230-1239 (1998)), fish (e.g., C. Lyons et al., J. Biol. Chem., 268, 21351-21358 (1993); K. Murata et al., Dev. Biol., 127, 9-17 (1995)), mice (e.g., S. Shimizu et al., J. Biol. Chem., 258, 5858-5863 (1983)), rabbits (e.g., V. Lee et al., J. Biol. Chem., 268, 12412-12417 (1993)), frogs (e.g., J. Yang et al., Dev. Growth Differ., 39, 457-467 (1997)); H. Kubo et al., Dev. Growth Differ., 39, 405-417 (1997)), humans (e.g., M. Chamberlin et al., Proc. Nat'l Acad. Sci. USA, 87, 6014-6018 (1990)), dogs, cats and primates. See J. Harris et al., J. Seq. Map, 4, 361-393 (1994), for a review of the ZPC gene family in vertebrates; and J. Harris et al., DNA Seq., 4, 361-393 (1994), for a review of zona pellucida genes and cDNAs from mammalian species. Zona pellucida protein can be obtained from an animal's ovaries or from an egg cell of an animal and/or the surrounding extracellular matrix and tissue, for example from an oocyte or unfertilized egg of an animal at any stage of development. Synthetic or recombinant zona pellucida protein can incorporate all or an immunogenic portion of the amino acid sequences of a zona pellucida protein derived from any organism having a zona pellucida.

In a preferred embodiment of the immunogenic composition, the zona pellucida protein used in the composition or encoded by the polynucleotide used in the composition is a porcine zona pellucida protein, preferably a total porcine zona pellucida protein, which can be obtained from pig ovaries. A total porcine zona pellucida protein preparation includes all three major porcine zona pellucida proteins: pZP1, pZP3α and pZP3β. pZP3α and pZP3β each have reported molecular weights of about 55 kD, and pZP1 has a reported molecular weight of about 82 kD. The amino acid sequences of these three proteins are known (J.D. Harris et al., DNA Seq., 4, 361-393 (1994)). Other reported pZP proteins are believed to be degradation products of pZP1. The immunogenic

composition can, alternatively, contain fewer than all the major porcine zona pellucida proteins; for example it can contain pZP3α and pZP3β but not pZP1.

In another preferred embodiment of the immunogenic composition, the zona pellucida protein used in the composition or encoded by the polynucleotide used in the composition is an avian zona pellucida protein, preferably a total avian zona pellucida protein, which can be obtained from the perivitelline membrane of bird eggs. A total avian zona pellucida protein preparation includes a plurality immunoreactive proteins, some of which have been identified in chickens as gp42 and gp97 (Y. Takeuchi et al., Eur. J. Biochem. 260:736-742 (1999)), and gp34 and gp95 (M. Waclawek et al., Biol. Reprod. 59:1230-1239 (1998)). Example VI, below, identifies immunoreactive chicken proteins having molecular weights of 70 kD, 40 kD and 35 kD. The immunogenic composition of the invention can, alternatively, contain fewer than all the avian zona pellucida proteins; for example it can contain the 40 kD and the 35 kD proteins but not the 70 kD protein.

In yet another preferred embodiment of the immunogenic composition, the zona pellucida protein used in the composition or encoded by the polynucleotide used in the composition is a total fish zona pellucida protein obtained from fish oocytes, eggs or liver tissue (C. Lyons et al., J. Biol. Chem. 268:21351-21358 (1993); K. Murata et al., Dev. Biol. 127, 9-17 (1995)).

It is to be understood that the immunogenic composition of the invention can contain or encode zona pellucida proteins derived from more than one source. For example, an immunogenic composition can include both avian zona pellucida protein, which is readily available and relatively inexpensive to isolate, and porcine zona pellucida protein, which is more difficult and expensive to obtain. The relative amounts of various zona pellucida proteins used in a protein vaccine of the invention depend on the nature of the animal being vaccinated and the immunogenic response generated in the animal by the vaccine. In the polynucleotide vaccine of this aspect of the invention, nucleic acid sequences encoding different zona pellucida proteins can be delivered on the same vector, or on different vectors. It should be further understood that a

vector used in the polynucleotide vaccine of the invention can include multiple copies of a single nucleic acid sequence encoding a zona pellucida protein.

Purity of a zona pellucida protein or glycoprotein can be evaluated analytically using a combination or series of two-dimensional sodium dodecyl sulfate polyacrylamide gels (SDS-polyacrylamide gel electrophoresis, or SDS-PAGE) with silver staining, Coomassie Blue staining, and Western blot analysis, as described in the following Examples. Glycoproteins typically migrate electrophoretically in gels as broad smears rather than narrow bands, as a result of the variable levels of negative charge present in the constituent oligosaccharide chains. For example, a "substantially pure" total zona pellucida protein preparation isolated from pig ovaries migrates as two distinct smears in the gel electrophoretic experiments (one smaller smear representing pZP1, and one larger smear representing pZP3α and pZP3β), and shows immunological reactivity in Western blot analysis using a polyclonal antibody raised in rabbits to highly purified total porcine zona pellucida protein. In a substantially pure zona pellucida protein preparation, there are no detectable contaminating proteins. The absence of detectable contaminating proteins is determined by demonstrating that there are no proteins in the preparation that have electromigration patterns different from those exhibited by the zona pellucida proteins as determined by two-dimensional SDS-PAGE (silver-stained) or Western blot analyses of two-dimensional SDS-PAGE gels.

An immunogenic fragment of a zona pellucida protein or glycoprotein is a peptide fragment, preferably a glycosylated peptide fragment, that elicits an immune response in a subject to which it is administered. An immune response includes either or both of a cellular immune response or production of antibodies, and can include activation of the subject's B cells, T cells, helper T cells or other cells of the subject's immune system. For example, an immune response is evidenced by a detectable anti-ZP antibody level in the subject using ELISA substantially as described in Example II.

Immunogenicity of the zona pellucida fragment can be determined, for example, by administering the adjuvanted candidate fragment to the subject, then observing of the associated immune response by analyzing anti-

ZP fragment antibody titers in serum. Alternatively or additionally, histochemical analysis of the ovaries of a vaccinated organism can be performed. Evidence of ovarian pathology indicates that the candidate fragment is immunogenic. An immunogenic peptide fragment preferably contains more than seven amino acids, more preferably at least about 10 amino acids, most preferably at least about 20 amino acids.

The zona pellucida protein used in the immunogenic composition of the invention is preferably a naturally occurring protein but can also be a chemically, enzymatically, or recombinantly synthesized protein. A recombinant protein produced in a eukaryotic system is preferred because it is likely to be at least partially glycosylated (see, e.g., WO 9314786, published 5 August 1993). However, non-glycosylated recombinant or de-glycosylated wild-type zona pellucida proteins are also envisioned for use in the immunogenic composition of the invention.

Conjugated vaccine. Another embodiment of the immunogenic composition of the invention comprises a conjugated immunogenic composition; that is, a zona pellucida protein, or fragment thereof, conjugated to a carrier protein, preferably a carrier protein that is immunogenic in the intended recipient. Examples of immunogenic carrier proteins include keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and ovalbumin. Other examples of immunogenic carrier proteins include proteins that have be engineered to include selected epitopes, such as T cell, helper T cell or B cell epitopes of the species to be vaccinated or of another species, or viral, bacterial or parasitic epitopes.

When the carrier protein is immunogenic, the zona pellucida protein fragment conjugated thereto can be, but need not be, immunogenic itself. Optionally, the zona pellucida protein fragment can take the form of a "hapten" that, although able to interact with the products of an immune response, cannot itself stimulate a response. Haptens are incomplete immunogens but can be made fully immunogenic by coupling them to a suitable carrier molecule.

The zona pellucida protein, or fragment thereof, used in the conjugate can be a naturally occurring protein, a chemically or enzymatically

synthesized protein, or a recombinant protein. The zona pellucida protein, or fragment thereof, used in the conjugate is preferably, but need not be, glycosylated. In a glycosylated zona pellucida protein (i.e., a zona pellucida glycoprotein), the glycosylation pattern is preferably equivalent to the glycosylation pattern found on a native (i.e., naturally occurring) glycoprotein. The fragment is peptide fragment that preferably contains more than seven amino acids, more preferably at least about 10 amino acids.

Conjugation methods are well known in the art and can involve any of the various functional groups on the protein and the carrier (e.g., free sulfhydryls, amines, amides, carboxyl groups, and the like) that are capable of linkage and/or activation. Optionally, conjugation is achieved using a linker molecules, for example, a maleimide derivative. An example of a convenient conjugation system that utilizes maleimide activated carrier proteins is available from Pierce Chemical Company (Rockford, IL) under the tradename IMJECT.

The immunogenic composition of the invention preferably contains a zona pellucida protein that is heterologous with respect to the organism to which it is administered. However, conjugation allows effective administration of an immunogenic composition comprising a homologous zona pellucida protein, that is, a zona pellucida protein, or fragment thereof, that is derived from the species to which the immunogenic composition is intended to be administered. For example, in a homologous vaccine protocol according to the invention a conjugate formed by the covalent linkage of avian zona pellucida protein to a carrier protein is administered to a bird. The term "derived from" a particular species, as used in this context, means that the amino acid sequence of the zona pellucida protein, or fragment thereof, is substantially the same as the amino acid sequence of the naturally occurring zona pellucida protein obtained from that species. The zona pellucida protein may, optionally, be glycosylated; if glycosylated, it may exhibit a native or a non-native glycosylation pattern. Thus, the zona pellucida protein used in a zona pellucida protein-carrier conjugate prepared for and used in a homologous vaccination can be obtained by isolating the protein directly from ovarian tissue or an oocyte or egg of that species, or, alternatively, by chemical or enzymatic synthesis, or using a

recombinant expression system comprising DNA that encodes zona pellucida protein, or fragment thereof, of the species to which the immunogenic composition is intended to be administered. The DNA used in such a recombinant expression system is conveniently obtained from the intended species, or, alternatively, can be chemically or enzymatically synthesized.

Preferably, the carrier protein used in the conjugate is a heterologous protein (i.e., derived from a species that is different from, more preferably remote from, the species of the vaccinated organism), as heterologous carriers are expected to generate a stronger immune response than homologous carriers.

It should be understood that the immunogenic composition of the invention contemplates heterologous as well as homologous zona pellucida protein-carrier conjugates, together with associated methods comprising administration of the homologous and heterologous conjugates to organisms of the same or different species, respectively, as described in more detail below. Further, the invention should be broadly understood to include zona pellucida protein-carrier conjugates themselves, in addition to immunogenic compositions including said conjugates, and methods of administration and use of said conjugates and compositions, as further described herein.

In a particularly advantageous embodiment, the invention provides a dually or multiply functional conjugate comprising a zona pellucida protein, or fragment thereof, conjugated to at least one immunogenic carrier protein that is selected so as to elicit a desired, independently protective, immune response. A dually functional conjugate refers to a zona pellucida protein, or fragment thereof, conjugated to one independently protective carrier protein, and a multiply functional conjugate refers to a zona pellucida protein, or fragment thereof, conjugated to two or more independently protective carrier proteins, or to a single carrier protein or protein construct that is capable of eliciting two or more independently protective immune responses. The carrier protein is advantageously selected to elicit immunological protection against an infection or disease state to which the intended subject may be exposed.

Where a fragment of zona pellucida protein is utilized, it is preferably, but need not be, an immunogenic fragment, capable of generating an immune response when administered in an unconjugated form. Because it is coupled to an immunogenic carrier, a non-immunogenic fragment will nonetheless elicit an immune response from the organism to which it is administered.

An example of a dually functional conjugate according to the invention is a zona pellucida protein, or fragment thereof, conjugated to an immunogen that stimulates immunity to beak and feather disease virus. This conjugate can be administered to birds in a single vaccine to simultaneously cause both immunosterilization (or immunocontraception) and immunity to beak and feather disease virus. Similarly, zona pellucida protein, or fragment thereof, can be conjugated to viruses or viral cell wall components, such as, for example, those currently in use as an avian polyomavirus vaccine (e.g., an avian polyomavirus vaccine from Biomune, Lenexa, KS).

Polynucleotide vaccine. The polynucleotide vaccine of the invention contains one or more polynucleotides encoding one or more polypeptides comprising a zona pellucida protein or immunogenic fragment thereof. In a preferred embodiment, the encoded polypeptide also contains at least one T cell, helper T cell or B cell epitope. The epitope can be derived from the species to which the vaccine is to be administered, from the species that was the source of the zona pellucida protein or immunogenic fragment thereof, or from any other species, including a virus, bacterium, or parasite. T cell, helper T cell or B cell epitopes or epitope mimics have been identified for ZP proteins (Garza et al., J. Reprod. Immunol., February 1998, pp. 87-101). The use of epitopes derived from an immunogenic organism, such as a pathogenic parasite, is preferred. For example, the polynucleotide vaccine can encode a chimeric peptide comprising a T cell or helper T cell epitope from a parasite and a B cell epitope from a porcine or avian zona pellucida protein (Bagavant et al., Biol. Reprod., March 1997, pp. 764-770). A vaccine for use as an immunosterilant preferably contains a polypeptide that contains at least one T cell epitope (or a polynucleotide functionally encoding such a polypeptide), whereas a vaccine for

use as an immunocontraceptive preferably includes a polypeptide that encodes at least one B cell epitope (or a polynucleotide functionally encoding such a polypeptide).

The polynucleotide vaccine can include DNA, RNA, a modified nucleic acid, or any combination thereof. The polynucleotide encoding a zona pellucida protein or immunogenic fragment thereof can be supplied as part of a vector or as a "naked" polynucleotide. General methods for construction, production and administration of polynucleotide vaccines are known in the art, e.g. F. Vogel et al., *Clin. Microbiol. Rev.* 8:406-410 (1995). Polynucleotides can be generated by means standard in the art, such as by recombinant techniques, or by enzymatic or chemical synthesis.

A polynucleotide used in a polynucleotide vaccine of the invention is preferably one that functionally encodes a zona pellucida protein. A protein is "functionally encoded" if it is capable of being expressed from the genetic construct that contains it. For example, the polynucleotide can include one or more expression control sequences, such as *cis*-acting transcription/translation regulatory sequences, including one or more of the following: a promoter, response element, an initiator sequence, an enhancer, a ribosome binding site, an RNA splice site, an intron element, a polyadenylation site, and a transcriptional terminator sequence, which are operably linked to the coding sequence and are, either alone or in combination, capable of directing expression in the target organism. An expression control sequence is "operably linked" to a coding sequence if it is positioned on the construct such that it does, or can be used to, control or regulate transcription or translation of that coding sequence. Preferred expression control sequences include strong and/or inducible *cis*-acting transcription/translation regulatory sequences such as those derived from metallothionine genes, actin genes, myosin genes, immunoglobulin genes, cytomegalovirus (CMV), SV40, Rous sarcoma virus, adenovirus, bovine papilloma virus, and the like.

The coding and expression control sequences for the zona pellucida protein are preferably constructed in a vector, such as a plasmid of bacterial origin, a cosmid, episome, or a viral vector, for administration to a

target organism. A vector useful in the present invention can be circular or linear, single-stranded or double stranded. There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccine plasmids. A specific embodiment employs constructs using the plasmid pcDNA3.1 as the vector (InVitrogen Corporation, Carlsbad, CA). In addition, the vector construct can contain immunostimulatory sequences (ISS) that stimulate the organism's immune system. Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences coding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF) or interleukin-12 (IL-12). The cytokines can be used in various combinations to fine-tune the response of the organism's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to affect the organism's reproductive system.

Alternatively, the vector can be a viral vector, including an adenovirus vector, and adenovirus associated vector, or a retroviral vector. Preferably the viral vector is a nonreplicating retroviral vector such as the Moloney murine leukemia virus (N2) backbone as described by Irwin et al. (*J. Virology* 68:5036-5044 (1994)).

Adjuvanted composition. The immunogenic composition of the invention, whether it contains a protein (e.g., conjugated or non-conjugated zona pellucida protein) or polynucleotide, optionally includes an immunological adjuvant to enhance the immunological response of the subject to the protein immunogen. Examples of adjuvants include Freund's Complete Adjuvant, Freund's Incomplete Adjuvant, Freund's mycotoxin-free adjuvant, aluminum hydroxide, EQUIMUNE (a deproteinized highly purified cell wall extract derived from non-pathogenic *Mycobacteria* spp., Acemannan (a long-chain polydispersed β(1,4) linked mannan polymer interspersed with O-acetylated groups, permulum, or an adjuvant comprising an immunostimulant such as synthetic trehalose dicorynomycolate (STDCM). The vaccine can also optionally include an oil, such as squalene oil, drakeol, or vegetable oil, which can also have an adjuvant effect (see P. Willis et al., J. Equine Vet. Sci., 14, 364-370 (1994)). It should be noted, however, that companion birds exhibit a

sensitivity to oil-based adjuvants, which cause necrotic granulomas at the site of administration. Reptiles are also sensitive to oils. Therefore, a non-oil-based adjuvant, such as aluminum hydroxide, is preferred for use in companion birds and reptiles. An adjuvant comprising synthetic trehalose dicorynemycolate, squalene oil, and a surfactant such as lecithin is preferred for use in organisms that are not sensitive to oils. Lecithin typically includes phosphatidyl choline.

When an oil adjuvant is used, homogenization of the adjuvant, such as Freund's adjuvant, with the aqueous zona pellucida protein or polynucleotide solution can be accomplished using any convenient means known in the art, such that the oil disperses within the aqueous solution to form an oil in water emulsion. Oil droplet sizes of about 200 nm or less are particularly preferred as they produce a more uniform and stable suspension. A particularly preferred immunogenic composition comprises a predetermined amount of zona pellucida protein or polynucleotide and a pharmaceutically acceptable immunogenic level of adjuvant in an emulsion containing about 10% oil phase and about 90% aqueous phase.

Non-adjuvanted composition. In another embodiment of the invention, the immunogenic composition, whether it contains a conjugated or a non-conjugated zona pellucida protein or a polynucleotide, contains no adjuvant; essentially the immunogenic composition of this embodiment is an aqueous zona pellucida protein or polynucleotide solution that delivers the intended amount of zona pellucida protein or polynucleotide to the recipient.

Administration of the immunogenic composition. The invention further includes a method for administering the immunogenic composition of the invention to an oocyte-producing organism such as a bird, a rabbit, a fish, a reptile, an amphibian, an insect, an arachnid or a parasite. Birds include but are not limited to free-ranging birds and domesticated birds, including food birds such as chickens, turkeys, and waterfowl, and companion birds in the order Psittaciformes, Passeriformes, Columbiformes, Falconiformes, such as budgerigars, canaries, finches, cockatiels, lovebirds, pigeons, doves and hawks. Fish include but are not limited to koi, goldfish and cichlids. Reptiles include but are not limited to lizards, snakes, chelonians (turtles) and crocodilians.

Amphibians include but are not limited to frogs, toads, and salamanders. Insects include but are not limited to roaches, ants, wasps, flies, hornets, mites, fleas, and ticks; arachnids include but are not limited to spiders and scorpians. Parasites include animal and plant parasites, and include, for example, intestinal parasites such as nematodes.

Preferably, the zona pellucida included in the immunogenic composition or encoded by the polynucleotide included in the immunogenic composition is heterologous with respect to the organism to which it is administered. For example, a preferred immunogenic composition for administration to birds contains porcine zona pellucida, which is heterologous with respect to the bird. Administration of a homologous zona pellucida protein or polynucleotide encoding a homologous zona pellucida protein is not excluded but it is not a preferred method, since the immune response generated by a homologous protein is typically substantially reduced in comparison to a heterologous protein. However, if it is conjugated to an immunogenic carrier protein as described above, a homologous zona pellucida protein is likely to generate a more significant immune response, and administration of such a conjugate represents a preferred embodiment of the method of the invention.

The immunogenic composition is administered in a manner and an amount effective to cause the desired response in the subject. For example, to treat an egg-bound bird, reptile or fish, with a protein vaccine of the invention, the immunogenic composition is preferably administered in the form of a plurality of doses (typically about 0.25 mL), each dose containing zona pellucida protein, or an immunogenic fragment thereof, in an amount of about 10 μg to about 2 mg, more preferably about 50 μg to about 400 μg. A polynucleotide vaccine is preferably administered in one or more doses containing the plasmid, viral vector or naked polynucleotide in an amount of about 5 μg to about 100 μg. One of skill in the art can readily determine a suitable dosage for a particular organism, depending on the nature, size and overall health of the organism, as well as the condition to be treated. An immunostimulant such as STDCM is optionally present in a per dose amount of about 10 μg to about 5 mg, preferably

in an amount of about 50 μg to about 3.5 mg, more preferably in an amount of about 1 mg to about 3 mg.

When administered to a bird, fish or reptile, the immunogenic composition preferably contains AlOH as an adjuvant, or contains no adjuvant at all.

The immunogenic composition is typically administered by way of intramuscular injection. However, other forms of administration are also contemplated, including subcutaneous or intradermal administration, oral administration, as by food or water, topical administration, including transdermal administration, aerosol administration, cloacal or vaginal administration, intracoelomic administration, intranasal administration, transconjunctival administration, including the use of eye drops. For fish, the immunogenic composition can be administered by immersing the fish in a solution containing the zona pellucida protein and the desired adjuvant, if any. In addition, liposome-mediated, microsphere-mediated, and microencapsulation systems are all included as delivery vehicles for the immunogenic composition of the present invention.

Initial administration of the vaccine, for example by injection, is preferably followed by two or more administrations, such as booster injections, at one to two week intervals, although the boosters can be administered from about 90 days to several months following the previous vaccination.

Uses of the vaccine. The immunogenic composition of the invention is used in oocyte-producing organisms such as birds, fish, rabbits, reptiles, amphibians, insects, arachnids and parasites to affect the reproductive systems of these organisms. Administration of the immunogenic compositions allows for control of reproduction, treatment of reproductive diseases or disorders, and/or alteration of the behavior of the organism.

Control of reproduction in an organism in accordance with the invention can take the form of either immunocontraception or immunosterilization. Immunosterilization means permanent, irreversible infertility, in contrast to immunocontraception wherein infertility is temporary or transient, and reversible. Immunocontraception and immunosterilization are

both dependent on the immune response of the subject, but immunosterilization is typically the result of ovarian pathology caused by vaccine administration and high titers of anti-ZP antibodies, as evidenced by, for example, total destruction of the zona pellucida proteins and/or influx of leukocytes into the follicles. Reducing the number of boosters can lead to a reduced immune response which results in immunocontraception (i.e., infertility that is temporary and reversible) instead of immunosterilization.

Reproductive disorders in birds and reptiles that can be treated or prevented in accordance with the invention include, but are not limited to, egg-binding disease, dystocias, egg-related peritonitis, oophoritis, neoplasias of the reproductive tract, prolapsed oviduct and cloaca, salpingitis, metritis, oviduct impaction, cloacal problems, cystic hyperplasia, ectopic egg formation, and chronic egg laying. Reproductive disorders in fish that can be treated in accordance with the invention include, but are not limited to egg-binding disease, dystocias, egg-related peritonitis, oophoritis, salpingitis, oviduct impaction and ectopic egg formation. Reproductive disorders in rabbits that can be treated in accordance with the invention include, but are not limited to dystocia, peritonitis, neoplasias of the reproductive tract, neoplasias of the mammary glands, metritis and cystic hyperplasia.

It is further anticipated that the immunogenic composition of the invention can be used as a growth stimulant in fish, thereby increasing the rate of weight gain and the efficiency of food conversion.

Moreover, female birds, reptiles and rabbits can exhibit undesirable behavior as a result of reproductive activity or reproductive problems including excess vocalization (birds), aggressiveness, biting, destruction of the local environment (birds, rabbits), and feather mutilation (birds). It is anticipated that the immunogenic composition of the invention can be used to treat or prevent these behavioral disorders.

It is also anticipated that the immunogenic composition of the invention can be used as a pest management tool, for example, to inhibit reproduction of pests by spraying the immunogenic composition onto a pest population, or by use in baited traps, which can include oral baits or baits with

an aerosol. Examples of pest populations that can be controlled thereby include ants, particularly fire ants, flies, mosquitoes, ticks, mites and fleas. Reproduction of fire ants, for example, can be accomplished by baiting with food that contains one or more zona pellucida proteins or by baiting with a protozoan engineered to contain a recombinant zona pellucida construct that can be expressed in the protozoan or the fire ant or both. Because food digestion in ants such as fire ants is facilitated by the internal presence of protozoans, protozoans can be used as vehicles for administration of the zona pellucida proteins or immunogenic fragments thereof to the ants.

EXAMPLES

Advantages of the invention are illustrated by the following examples. However, the particular materials and amounts thereof recited in these examples, as well as other conditions and details, are to be interpreted to apply broadly in the art and should not be construed to unduly restrict or limit the invention in any way.

Example I. Isolation of Porcine Zona Pellucida and Extraction of pZP Proteins

Buffers. Saline buffer (40 L) was made by addition 4 L of the following solution: 0.9% NaCl, 0.01 M dibasic sodium phosphate, 0.01 M monobasic sodium phosphate, and 0.002 M sodium citrate dihydrate, pH 7.2, in triple distilled water, to 36 L of triple distilled water. Tris buffer (3L) was made by adding 484 g Tris base, 119 g ethylenediaminetetraacetic acid (EDTA), 47 g sodium citrate dihydrate and 16 g sodium azide to 3L of triple distilled water, then adjusting the pH to 7.9. Tris detergent buffer (1L) was made by combining 2 mL of NP-40 (Cat. No. N-6507, Sigma Chemical Co., St. Louis, MO) with 998 mL Tris buffer.

Other materials. The oocyte purification apparatus consisted of three chambers. Each chamber consisted of a stainless steel wire mesh container

(Home Depot) suspended inside a buffer container set on an orbital shaker (shown in Fig. 1) or a rotary washing system with an internal agitator. The pore size of the wire mesh used to form the wire mesh containers in the first, second, and third chambers was 1000 μm, 500 μm, and 150 μm, respectively. Tubing connecting the chambers allowed fluid transfer from the buffer space external to the wire mesh of one chamber to a collection or holding carboy, or, alternatively, to the inside of the next succeeding downstream wire mesh container in a continuous flow process, as shown in Fig. 1. Peristaltic pumps are used to effect fluid movement within the tubing between chambers (as shown in Fig. 1) or between the chambers and any collection carboys used (not shown in Fig. 1).

Pig ovaries were obtained from pig slaughterhouses.

Zona pellucida isolation. Porcine ovaries (5-6 lbs.) were twice ground through a commercial meat grinder (Hobart), and the homogenate was collected. The homogenate and grinder were rinsed with 4L of saline buffer, and the homogenate solution was placed in the wire mesh container of the first chamber of the purification apparatus. The three buffer containers of the purification apparatus were filled with saline buffer. The shakers were operated at an agitation cycler rotation speed of about 20 revolutions per minute during the oocyte purification process. Periods of rotary agitation were alternated with periods of fluid removal from the region surrounding the mesh container. Filtered oocytes, together with a small amount of tissue, passed through the 1000 μm mesh and were thus pumped from the buffer space of the first chamber into a collection carboy or into the wire mesh container in the second chamber. In purification procedures making use of a collection carboy, the filtered oocytes are subsequently pumped into the wire mesh container in the second chamber. With rotary agitation and new saline buffer addition, the oocytes were then passed through the 500 μm mesh of the wire mesh container of the second chamber while the fibrous tissue remained in the mesh container. The oocytes and saline buffer were then pumped from the buffer space of the second chamber into a collection carboy or directly into the 150 μm wire mesh container in the third chamber. Rotary agitation was continued in the third

chamber and the solution surrounding the wire mesh (containing the oocytes) was removed.

The solution containing the oocytes was then passed over a 75 μm screen ($1^3/_4$ inches or 2½ inches in diameter). The oocytes were collected on the 75 μm screen and were then backwashed into a 100 mL beaker using Tris buffer. The 100 mL solution was divided into 2 x 50 mL vials and homogenized at 15,000 rpm for 3 to 5 minutes in a Powergam 700D (Fisher) homogenizer.

The zona fragments were then poured onto a $1^3/_4$ inches or 2½ inches diameter, 0.040mm (40μm) filter screen and washed with Tris detergent buffer. The zona fragments were removed from the screen by backwashing with Tris detergent buffer into a small polypropylene beaker, then incubated at 4°C with constant mechanical stirring to dissociate any undesired proteins, such as albumin. The zona material is preferably handled in polypropylene or siliconized glass beakers to prevent adherence to surfaces which results in loss of the material.

After incubation and stirring, the zona fragments were again poured a $1\ ^3/_4$ inch diameter, 0.040mm (40μm) filter screen and washed with Tris buffer to remove any protein contaminants. The zona fragments retained on the screen were collected by spooning or backwashing (using Tris buffer) into a small polypropylene beaker to a maximal volume of 25 mL. The beaker was covered and placed in a 75-76°C water bath and incubated for 20 minutes to solubilize the zona protein such that the temperature of the zona protein-containing solution was 73 ± 1°C.

After solubilization, the mixture was centrifuged at 21,000 rpm for 25 minutes or until a pellet was observed at the base of the tube. The supernatant was collected, and protein concentration was estimated. The supernatant was aliquoted (3mg/vial), lyophilized, and stored under N_2 gas in a desiccator at 4°C. Typically about 1.5 mg to 1.9 mg of highly purified pZP protein per pound of ovaries can be produced, amounting to about 10 mg on a daily basis. Previous techniques produced only about 200 - 300 μg quantities

over a two day period. It is anticipated that this harvesting technique of the present invention can be increased to produce even greater amounts.

Purity was demonstrated and confirmed using two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with Western blot analysis, silver staining, and, at times, Coomassie blue staining, using standard protocols. The preparation was tested for viral and bacterial contaminants at the Diagnostic Laboratory at the College of Veterinary Medicine at the University of Georgia.

Example II. Preparation of a ZP Vaccine

A pZP vaccine was prepared by homogenizing Freud's complete adjuvant with an aqueous antigen solution containing isolated pZP protein. The aqueous antigen solution contained the pZP protein preparation in saline or phosphate buffered saline (PBS) and Tween 80. When prepared for use in the vaccine composition, the aqueous composition typically contained 0.4% (vol/vol) Tween 80 and an amount of pZP calculated to yield the desired pZP dose. Vaccine volumes for the chickens were about 1 mL; the dose to be used for smaller birds is about 0.25 mL.

An avian ZP vaccine, for use in organisms other than chickens, can be prepared using the same procedure, but substituting avian ZP for porcine ZP.

Example III. Vaccination of Chickens with pZP Vaccine

Vaccinations. Four experimental SF leghorn chickens were vaccinated with 200 μg of pZP per dose (1 mL volumes) in a vaccine adjuvanted with Freund's complete adjuvant. The chickens were vaccinated with three injections administered at approximately two week intervals. Three unvaccinated control chickens were included in the trial. Under veterinary supervision, vaccinations were delivered to chickens intramuscularly in the deep

pectoral muscle. Booster injections were administered on the contra-lateral side. No pain or adverse reactions were observed at the injection sites.

Antibody titers. Blood was drawn from each chicken at the time of each of the three injections, and about three weeks following the last injection. Serum antibody titers (IgG) were determined using an enzyme linked immunosorbant assay (ELISA). Adjacent wells of a microwell plate were coated with 2 μg pZP, and incubated for 6 hours. The wells were then blocked with 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in TBST (Tris-buffered saline + 5% Tween-20) and incubated overnight. Wells were then loaded with the primary antibody (i.e., avian serum) in TBST at a 1:500 and 1:1,000 dilution and incubated for 4 hours. The wells were then washed and loaded with 50 μl of the secondary antibody (rabbit anti-chicken IgG) and incubated for 2 hours. Color change was observed after the addition of *p*-nitrophenyl phosphate for 30 minutes and the reaction terminated by the addition of 3 M NaOH. The optical density was read at a 405-492 nm range on a Spectramax spectrophotometer. The chickens' pre-immune serum served as the negative controls.

The ELISA trials (Fig. 2) revealed that there was a similar antibody profile in all four experimental chickens characterized by a significant rise in antibody titers between the initial injection and the first booster. Antibody levels remained high after the first and second boosters. These data clearly show that there is a significant immune response to the adjuvanted pZP vaccine. The rapidity of the effect suggests an IgM-mediated response in the chickens.

Egg laying. Eggs were counted beginning on the day of the first injection. After 13 weeks, the three control chickens had laid, as a group, 81 eggs, and the four experimental chickens had laid, as a group, only 4 (Fig. 3). An immediate reduction in egg production was observed in the experimental animals.

Example IV. Treatment of Egg-binding Disease in Chicken

A pZP vaccine (1 mL volume) containing 200 μg of pZP adjuvanted with Freund's complete adjuvant was administered intramuscularly as described in Example III to an hysterectomized chicken that had behavioral and physical abnormalities (broodiness, cloacal prolapse) consistent with ovulation. Within 24 hours of administration, cloacal prolapse was no longer evident and straining was eliminated.

Example V. Conjugated pZP Vaccine

Materials and Methods. A pZP-KLH conjugate was made using IMJECT® Maleimide Activated Keyhold Limpet Hemocyanin kit (Pierce Chemical Company, Rockford IL) using the method and materials provided with the kit substantially without modification. The instructions supplied with the IMJECT kit are hereby incorporated by reference in their entirety. Briefly, porcine zona pellucida protein (2 mg pZP) was reduced in 2-3 mL buffer (0.1 NH_4HCO_3, pH 8) by the addition of dithiothreitol (DTT, 1.5 mg/mL), then dialyzed against distilled water containing 10 mM EDTA (pH 7) to remove excess DTT. The reduced protein was lyophilized, then redissolved using between 200 μL to 500 μL conjugation buffer supplied in the Pierce kit to a concentration of between about 4 μg/μL to about 10 μg/μL pZP, respectively. KLH, activated according to the instructions of the kit (200 μl), was added to reduced pZP by mixing the two solutions, then the mixture was sparged with nitrogen gas for 2 minutes and left at room temperature for 2 hours to complete the conjugation. The conjugate was then dialyzed against Purification Buffer Prod. # 77159 available from Pierce Chemical Co. (Rockford, IL) (i.e., 0.083 M sodium phosphate, 0.9 M NaCl, pH 7.2, with stabilizers).

Per dose, the pZP-KLH conjugate (about 0.5-2 mg) was combined with adjuvant supplied by RIBI Immunochem Co. (Hamilton, MT)

comprising 2.5 mg STDCM, in about 1-4 mL drakeol oil. Homologous vaccination was effected by administering the vaccine to three pigs two to four times at 2-3 week intervals. Serum titers were evaluated, and ovarian histopathology was observed.

Results. The resulting conjugate is, presumably, in the form of pZP covalently linked, via a maleimide linker, at the free sulfhydryl (-SH) of one or more of the constituent cysteines to the free amine ($-NH_2$) of a KLH lysine or N-terminus. KLH is commonly used as a carrier protein to impart immunogenicity to covalently coupled haptens. In the current experiment, KLH was coupled to porcine zona pellucida protein (which is not a small-molecule "hapten") in an effort to induce an immune response in a homologous vaccination (i.e., vaccination of a pig). Typically, it is difficult to generate an immune response in a homologous vaccination protocol because the body does not treat the potential immunogen as "foreign" or "nonself" material.

The KLH-pZP conjugate was found to generate a strong immune response in the vaccinated pigs. Severe damage to the ovaries was observed, evidenced by massive infiltration of lymphocytes, neutrophils, and macrophages into the large follicles, consistent with irreversible sterilization. Interestingly, serum titers were lower than expected for this degree of ovarian pathology.

Example VI. Isolation of Avian Zona Pellucida Proteins

Perivitelline membranes were obtained from laid chicken eggs. Yolks were separated from whites by making a small hole in the egg and draining the albumin. The yolks were removed from the shell and the chalazae was removed and discarded. The perivitelline membranes (pvm) were either manually peeled away or punctured to drain the yolk, and the membranes were washed in sterile phosphate buffered saline solution. A tissue homogenizer (Powergen 700D) was used to homogenize the membranes. M. Waclawek et al. (Biol. Reprod., 59, 1230-1239 (1998)) reported a similar procedure for isolating perivitelline membranes from laid eggs that can also be used. Alternatively, perivitelline membranes can be isolated directly from ovarian follicles by

preparing granulosa cell sheets substantially as described by A. Gilbert et al, J. Reprod. Fertil., 50, 179-181 (1977)).

Purity was demonstrated and confirmed using one-dimensional and two-dimensional SDS-PAGE combined with Western blot analysis, silver staining, and, at times, Coomassie blue staining, using standard protocols. Proteins having molecular weights of 70 kD, 40 kD and 35 kD reacted with rabbit anti-pZP serum. Yolk and albumin controls did not react with rabbit anti-pZP serum. The 70 kD protein was easily washed away from the perivitelline membrane. The 35 kD protein was approximately twice as abundant as the 40 kD protein and both had strong reactivity to the anti-pZP serum. One or both of these proteins may to be homologous to mammalian ZP3 (also known as ZPC), according to published reports (Y. Takeuchi et al., Eur. J. Biochem., 260, 736-742 (1999); M. Waclawek et al., Biol. Reprod., 59, 1230-1239 (1998)).

Example VII. Ovarian Response in Fire Ants to Rabbit Anti-pZP Antibodies

Ovaries of fire ants were sectioned and stained with hematoxylin and eosin. This confirmed the architecture of the ovaries.

Further, the fire ant ovaries were immunostained with rabbit anti-pZP serum. Specifically, sections of tissues were heated in a 60°C oven for 20 minutes followed by two washes with xylene in order to remove all paraffin. The sections were then rehydrated through an ethanol series followed by incubation in a 9:1 methanol/H_2O_2 solution for 30 minutes to remove endogenous peroxidase. Nonspecific binding sites on the sections were blocked by incubating sections in 5% bovine serum albumin (BSA) dissolved in Tris buffered saline (TBS) containing 0.05% Tween 20 (TBST) overnight. Rabbits that had been vaccinated with highly purified pZP provided the source of the anti-pZP IgG. The serum was diluted 1:1,000 with TBST and 200 µl were applied to the tissue sections and incubated for one hour in a wet chamber. Negative control sections were treated with a similarly diluted normal rabbit serum. Sections were washed twice with TBS after incubation with primary

antibody followed by incubation with 200 µl of goat anti-rabbit IgG biotin conjugate at a 1:1,000 dilution for 30 minutes in a wet chamber. After incubation, the sections were washed twice with TBS and then treated with Extravidin peroxidase (1:100 in TBST, Sigma Chemical, St. Louis, MO) for 30 minutes in a wet chamber. Tissue sections were washed twice with TBS and treated with diaminobenzidine (DAB) for 2 minutes. Color reaction was stopped with running distilled water followed by counterstaining with Mayer's hematoxylin. Sections were then dehydrated through an ethanol series and permanently mounted. Observations were made with a Zeiss Axioscope microscope at 40 and 100 x magnification.

The immunocytochemistry showed that the anti-pZP reacted with the ovarian tissue of the fire ant queens, indicating that the fire ant ovaries contained a pZP-like target and suggesting that the ants would be affected by the zona pellucida vaccine.

Example VIII. Vaccination of Chickens with Mixed Avian ZP/pZP Vaccine

Vaccinations. Chickens are vaccinated with 200 µg of mixed zona pellucida protein (1:1, avian ZP:porcine ZP) per dose (1 mL volumes) in a vaccine adjuvanted with Freund's complete adjuvant. Avian ZP is isolated as in Example VI. The birds are vaccinated with three injections administered at approximately two week intervals. Under veterinary supervision, vaccinations are delivered to chickens intramuscularly in the deep pectoral muscle. Booster injections are administered on the contra-lateral side.

Antibody titers. Blood is drawn from each bird at the time of each of the three injections, and about three weeks following the last injection. Serum antibody titers (IgG) are determined using an enzyme linked immunosorbant assay (ELISA). Adjacent wells of a microwell plate are coated with 2 µg aZP, and incubated for 6 hours. The wells are then blocked with 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in TBST (Tris-buffered saline + 5% Tween-20) and incubated overnight. Wells are then loaded

with the primary antibody (i.e., avian serum) in TBST at a 1:500 and 1:1,000 dilution and incubated for 4 hours. The wells are then washed and loaded with 50 µl of the secondary antibody (rabbit anti-chicken IgG) and incubated for 2 hours. Color change is observed after the addition of *p*-nitrophenyl phosphate for 30 minutes and the reaction is terminated by the addition of 3 M NaOH. The optical density is read at a 405-492 nm range on a Spectramax spectrophotometer. The chickens' pre-immune serum serves as the negative control.

Egg laying. Eggs are counted beginning on the day of the first injection to evaluate reduction in egg production, providing direct evidence of effect on the chicken's reproductive system.

Example IX. Preparation of Protein Vaccine for Use in Companion Birds, Rabbits, Reptiles and Fish

Adjuvanted vaccine. A vaccine is prepared by mixing aluminum hydroxide (AlOH) with an aqueous antigen solution containing one or more isolated ZP protein e.g., avian ZP and/or pZP. The aqueous antigen solution contains the ZP protein preparation in saline or phosphate buffered saline (PBS) and Tween 80. When prepared for use in the vaccine composition, the aqueous composition typically contains 0.4% (vol/vol) Tween 80 and an amount of ZP calculated to yield a dose of about 10 µg to about 2 mg per vaccination.

Non-adjuvanted vaccine. A vaccine is prepared using an aqueous antigen solution containing isolated ZP protein(s), without an adjuvant. The aqueous antigen solution contains the ZP protein preparation in saline or phosphate buffered saline (PBS) and Tween 80. When prepared for use in the vaccine composition, the aqueous composition typically contains 0.4% (vol/vol) Tween 80 and an amount of ZP protein calculated to yield a ZP dose of about 50 µg to about 2 mg per vaccination.

Vaccine volumes for the adjuvanted and the non-adjuvanted vaccines depend on the size of the animal but are typically about 0.25 mL.

The complete disclosure of all patents, patent applications, and publications cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.